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Studies on Erythropoiesis. Part IV. Reticulocyte Response of Hypophysectomized and Polycythemic Rodents to Erythropoietin. (22911)

L. O. JACOBSON, E. GOLDWASSER, L. F. PLZAK, AND W. FRIED
Argonne Cancer Research Hospital, Departments of Medicine and Biochemistry, University of Chicago, Ill.

Erythropoiesis is reduced to a minimum in hypophysectomized rats within 3 weeks after removal of the pituitary(1,2). During this same interval, there is a rapid overall reduction in metabolic requirements(3), although the red cell mass remains within normal limits(4). For this reason, we have suggested that the animal in this state is comparable to one with a transfusion-induced polycythemia and as a consequence, erythropoiesis decreases. Administration of plasma obtained from normal or hypophysectomized animals made anemic by repeated bleeding or phenylhydrazine administration produces an increase in incorporation of Fe^{59} into red cells of the hypophysectomized recipients. The increased incorporation is 3 or more times greater than that in control hypophysectomized animals that are given normal plasma or saline.

The effect of age at the time of hypophysectomy and the effect of transfusion-induced polycythemia upon erythropoiesis are described in this paper. In addition, the reticulocytic response of hypophysectomized rats and polycythemic mice to "anemic plasma" is also reported.

Materials and methods. Anemic and control plasmas were prepared according to the methods described in Part III(5) of this series. *Hypophysectomized rats of 2 age groups.* In one experiment, 30 Sprague-Dawley male rats, 26 to 28 days of age, were studied. The average body weight was 100 g. Reticulocyte determinations were made on all rats the day before 20 were hypophysectomized and the other 10 sham-operated. Thereafter, reticulocyte values were obtained daily from 10 hypophysectomized and 5 sham-operated rats on alternate days. This was continued for 7 days and then at less frequent intervals. In the second experiment,

conducted similarly, the rats were 2 months old with an average weight of 175 g. *Hypophysectomized recipients of anemic plasma.* Four days after hypophysectomy, Sprague-Dawley rats, each weighing about 200 g, were divided randomly into 3 groups, of 5 animals each. Animals in group 1 received no injections (untreated controls); those in group 2, "anemic" rat plasma; and those in group 3, normal rat plasma. Two-cc doses of plasma were injected daily into the tail vein on 3 consecutive days. Reticulocytes were counted prior to the first plasma injection and daily thereafter for 6 days. *Preparation of polycythemic recipients.* CF No. 1 mice weighing 23 g, were made polycythemic by intraperitoneal injections of 75 to 80% concentration of washed homologous red cells suspended in saline. The suspension was given in 0.5-cc doses on successive days and once again 2 days later. Hematocrit determinations were made thereafter at frequent intervals, and red cell injections were repeated when necessary to maintain hematocrit readings of about 70 to 75%. At about 6 days after the first injection, when no reticulocytes were found in the peripheral blood smear, the mice were used for the anemic plasma injection experiments. *Polycythemic recipients of anemic plasma.* Forty-five polycythemic mice were divided into 3 groups of 15 mice each. Those in group 1 received anemic rat plasma; those in group 2, normal rat plasma; and those in group 3, saline. The plasma or saline was injected intravenously for 4 successive days in doses of 0.5 cc. Beginning on fifth day, injections were given every other day until 11th day. In a separate but otherwise identical experiment, blood volume determinations were done on the 3 groups described above as well as on 5 normal mice that received saline and on 5 normal mice that received neither saline nor plasma. In another experiment, intravenous injections of plasma or saline were begun on the same day that mice were given the first intraperitoneal injection of red cells. Plasma was given for 19 days (5 days/week) and the red cell suspension at intervals sufficient to produce and maintain a polycythemic condition. Three groups of 10 mice each were studied. Those in group 1 received anemic rabbit plasma; those in group 2, normal rabbit plasma; and those in group 3, saline. Reticulocyte counts were made frequently throughout the 19-day period of study. In yet another study, doses of 0.5 cc normal or anemic rabbit plasma were given intraperitoneally at the same time that red cells were being given, by the same route, to each of 10 mice in 2 groups. The 10 mice in the third group received saline. Plasma injections were given for 16 days (5 days/week) and the red cells as necessary to produce and maintain a polycythemia. Hematocrit determinations and reticulocyte counts were made on 5, 8, 12, and 15 days following the first plasma and red cell injections.

All hematologic studies were made on blood drawn from tail vein and were limited, for purposes of this communication only, to hematocrits and reticulocytes. Blood for hematocrit determinations was collected in heparinized capillary tubes and spun in a microhematocrit centrifuge.* Reticulocytes were counted by the direct smear method, using brilliant cresyl blue without counterstain. When no reticulocytes were found in 10×10^6 red cells, the values were considered to be 0.000%. Blood volumes were determined according to Cr^{51} red cell labeling technic of Sterling and Gray(6). This was modified somewhat for the mice. The radioactivity of each sample was counted in a well-type scintillation counter using 0.1 cc of donor-tagged blood cells in 0.9 cc of distilled water as a standard. Hematocrit determinations were made at the time that blood for blood volume determinations was collected to permit calculation of the red cell mass as well as that of plasma volume.

For histologic studies, polycythemic mice were divided into 4 groups. Group 1 (control), consisted of polycythemic mice that had received neither plasma nor saline. Group 2 consisted of polycythemic mice that had received anemic rabbit plasma. Animals in group 3 had received normal rabbit plasma, and those in group 4, saline. Five mice in

* International Hematocrit Centrifuge, A. S. Aloe Co., St. Louis, Mo.

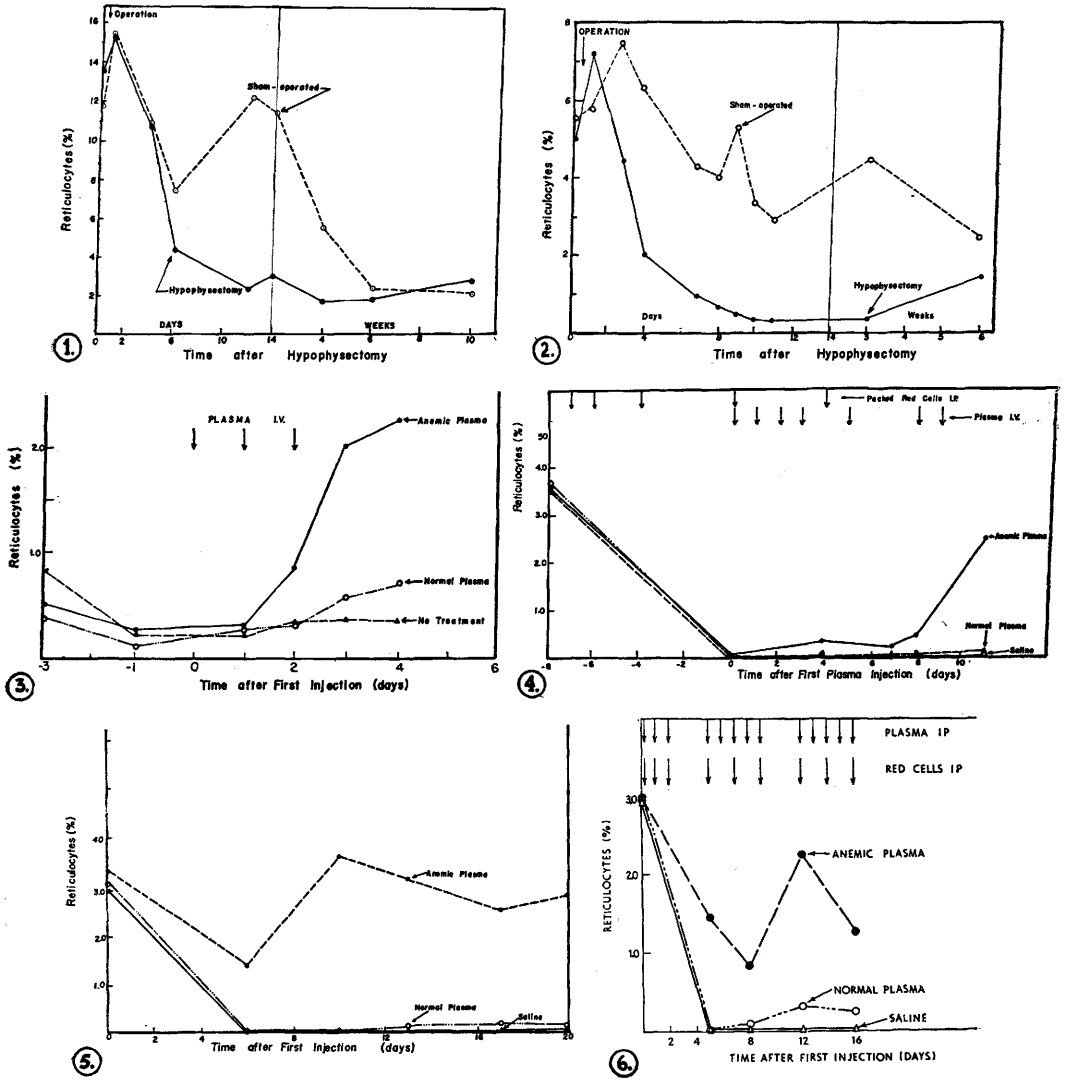

FIG. 1. Effect of hypophysectomy on reticulocyte values of 26- to 28-day-old rats (avg wt, 100 g).

FIG. 2. Effect of hypophysectomy on reticulocyte values of 52-day-old rats (avg wt, 175 g).

FIG. 3. Effect of 2-cc intrav. inj. of anemic plasma compared with that of normal rat plasma on reticulocyte values of hypophysectomized rats (avg wt, 200 g). Injections were started on the 7th day after hypophysectomy.

FIG. 4. Effect of 7 0.5-cc intrav. injections of anemic rat plasma compared with that of normal rat plasma on reticulocyte values of mice that had a transfusion-induced polycythemia.

FIG. 5. Effect of repeated 0.5-cc injections of anemic and normal rabbit plasma on reticulocyte values of mice that were given first intrav. inj. of plasma on same day that transfusions for polycythemia were initiated.

FIG. 6. Effect of repeated 0.5-cc intrav. injections of anemic and normal rabbit plasma on reticulocyte values of mice that received intraper. injections of plasma beginning on same day that transfusions for polycythemia were initiated.

each group were sacrificed 8 days after the first of 6 daily intravenous injections of 0.5 cc plasma or saline. The hematocrit was maintained at about 70% throughout this experimental period. The mice were killed by cervical fracture, and samples of liver, spleen, and bone marrow (femur) were taken for examination. The tissues were fixed in Zen-

TABLE I. Effect of Anemic Plasma on Blood Volume of Normal and Polycythemic CF No. 1 Mice.

Treatment	Wt (g)	Hematocrit (%)	Total blood vol (cc)	Total blood vol % body wt	Red cell mass (cc)	Plasma vol (cc)
Normal	23	54	1.46	6.3	0.79	0.67
Normal saline	24.6	55.5	1.59	6.4	0.88	0.71
Polycythemic saline	23.9	76.4	2.43	10.1	1.85	0.57
Polycythemic normal plasma	24.4	77	2.58	10.5	1.98	0.59
Polycythemic anemic plasma	24.8	74	2.23	9.0	1.65	0.58

ker-Formol, embedded in nitrocellulose, cut at 6 μ, and stained with hematoxylin-eosin-azure.

Results. Effect of age at time of hypophysectomy. The reticulocyte values of 26- to 28-day-old rats were about 13% on the day before hypophysectomy. During the first 5 days thereafter, the reticulocyte values of hypophysectomized and sham-operated rats were comparable. By 6 days, however, the reticulocyte values of the hypophysectomized rats were appreciably lower than those of sham-operated animals and, as indicated in Fig. 1, they continued to fall until the minimal value of 1.7% was reached by the 4th post-operative week. The number of reticulocytes in sham-operated rats fell more slowly, but by 6 weeks, the values of both groups were comparable and stabilized at 2.5%. Reticulocyte values of 2-month-old rats were about 5.5% before hypophysectomy. The reticulocytes dropped from the pre-operation value to 2% at 4 days afterwards in the hypophysectomized rats and continued to decline, reaching a minimum of 0.3% by 10 days. They remained at this level for 3 weeks, then gradually rose to 1.7% by 6 weeks The per cent of reticulocytes in sham-operated animals very gradually declined to 2.5 by 11 days after surgery and remained essentially unchanged thereafter (Fig. 2).

Effect of anemic rat plasma on hypophysectomized rats. As indicated in Fig. 3, the reticulocyte values of the hypophysectomized rats injected with anemic plasma rose to 2.3% by the fourth day after the first injection. Values for normal plasma controls reached a maximum of 0.7% whereas those for untreated controls remained at the baseline (Fig. 3).

Effect of anemic plasma on polycythemic mice. By 1 day following 4 daily injections of anemic rat plasma, the reticulocyte values of polycythemic mice were 0.4% as compared with 0.000% for mice that received similar injections of normal plasma or saline. By 11 days following repeated injections, the reticulocyte values of the group receiving anemic plasma were 2.3%, whereas the reticulocyte values of mice receiving normal plasma or saline were less than 0.001% (Fig. 4). Simultaneous Fe^{59} red cell incorporation studies and reticulocyte counts were conducted in this experiment. On the day that the reticulocytes of the polycythemic anemic plasma recipients were 0.4% and the normal plasma and saline recipients were 0.000%, the Fe^{59} red cell incorporation data failed to show any significant difference between the groups. When reticulocytes reached 2.6% however, the Fe^{59} red cell incorporation studies showed a value more than twice that of the saline or normal plasma controls but failed to show any significant difference between the latter groups.

A definite increase in red cell mass was found in the polycythemic mice that received anemic plasma, normal plasma, or saline (Table I). The plasma volume was decreased moderately in these polycythemic animals as compared with the untreated normal controls. As was expected, no significant difference in red cell mass was noted between the 3 polycythemic groups.

It is worthy of note that a total of only 4 injections of anemic plasma or normal plasma was given in this experiment and that less than 0.001% reticulocytes were observed in normal plasma- and saline-injected groups. In the other experiments described below, many more injections of anemic and normal plasma were given and a slight increase in

reticulocytes above the baseline was invariably observed in the normal plasma-injected animals.

In another experiment, the reticulocyte values of the recipients were 1.3% 6 days after first intravenous injection of anemic rabbit plasma and the first intraperitoneal injection of red cells, whereas these values were 0.000% in mice that received normal plasma or saline. By 10 days, when the reticulocyte values of mice that were given normal plasma or saline were still 0.000% the reticulocytes of the mice that received anemic plasma reached a peak of 3.7%. No further increase in number of reticulocytes occurred in mice receiving anemic plasma, even though the injections were continued for 20 days. A slight increase in reticulocyte values, (about 0.1%) however, occurred in mice receiving normal plasma by 20 days as compared with 0.000% in mice receiving saline (Fig. 5). Injections of anemic plasma given intraperitoneally at the same time that red cell suspensions were injected via the same route produced responses similar to those described above (Fig. 6). By 5 days after the first injections of plasma and red cells, the reticulocyte values of mice that received anemic plasma were 1.4% as compared with 0.000% for mice that had been given normal plasma or saline. By 12 days, the reticulocyte values of anemic plasma-treated animals had risen to 2.2% while those of animals that had been given normal plasma reached 0.25%. The saline recipients remained reticulocyte-free through the 16-day period of observation.

Histologic findings. Histologic differences between polycythemic mice that received anemic plasma and those that received saline or no injection were obvious at autopsy. The spleens of animals that had received anemic plasma were at least twice the size of those from saline-treated or control animals. The spleens from the polycythemic mice that had been given normal plasma were larger than those from the saline or "no injection" controls but smaller than those from the anemic plasma group.

Histologically, the 4 groups were clearly distinguishable as follows: 1) Anemic plasma-treated polycythemic recipients. Islands of ectopic erythropoiesis in the portal triads of liver were easily distinguishable. Evidence of erythropoiesis was scattered throughout the bone marrow. The red pulp of spleen was packed solidly with erythropoietic tissue (Fig. 7, D). The white pulp was sparsely populated with lymphocytes. 2) Normal plasma-treated polycythemic recipients. Definite islands of erythropoietic activity were found in the portal triads of liver but were less conspicuous than observed in liver of recipients of anemic plasma. The red pulp of spleen had definite areas of erythropoietic proliferation as shown in Fig. 7, B. The amount of erythropoietic tissue in the red pulp was clearly less than that observed in recipients of anemic plasma. 3) Saline-control polycythemic recipients. No indications of ectopic erythropoiesis in liver, no significant evidence of erythropoiesis in bone marrow was seen. The marrow consisted primarily of megakaryocytes and granulocytes. As indicated in Fig. 7, C, the red pulp of spleen was relatively acellular and there was no definite evidence of erythropoiesis. The white pulp stood out sharply in contrast. 4) "No injection" control polycythemic mice. Histologic findings in liver, bone marrow, and spleen in this group were identical with those for saline-injected control mice. A section of the spleen from such a mouse is shown for purposes of comparison in Fig. 7, A.

Discussion. In Part III(5) of this series we have attempted to explain the mechanism whereby hypophysectomy, transfusion-induced polycythemia, and related experimental conditions alter red cell production. We have also tentatively proposed a theory to explain the mechanism by which erythropoietin is produced and by which erythropoiesis is controlled. This communication is largely designed to show that the data on reticulocyte production and the histologic evidence of erythropoiesis in hemopoietic tissue support the findings in the preceding paper in which the incorporation of Fe^{59} into the red cell was utilized to study the state of erythrocyte production as well as to measure the effects of

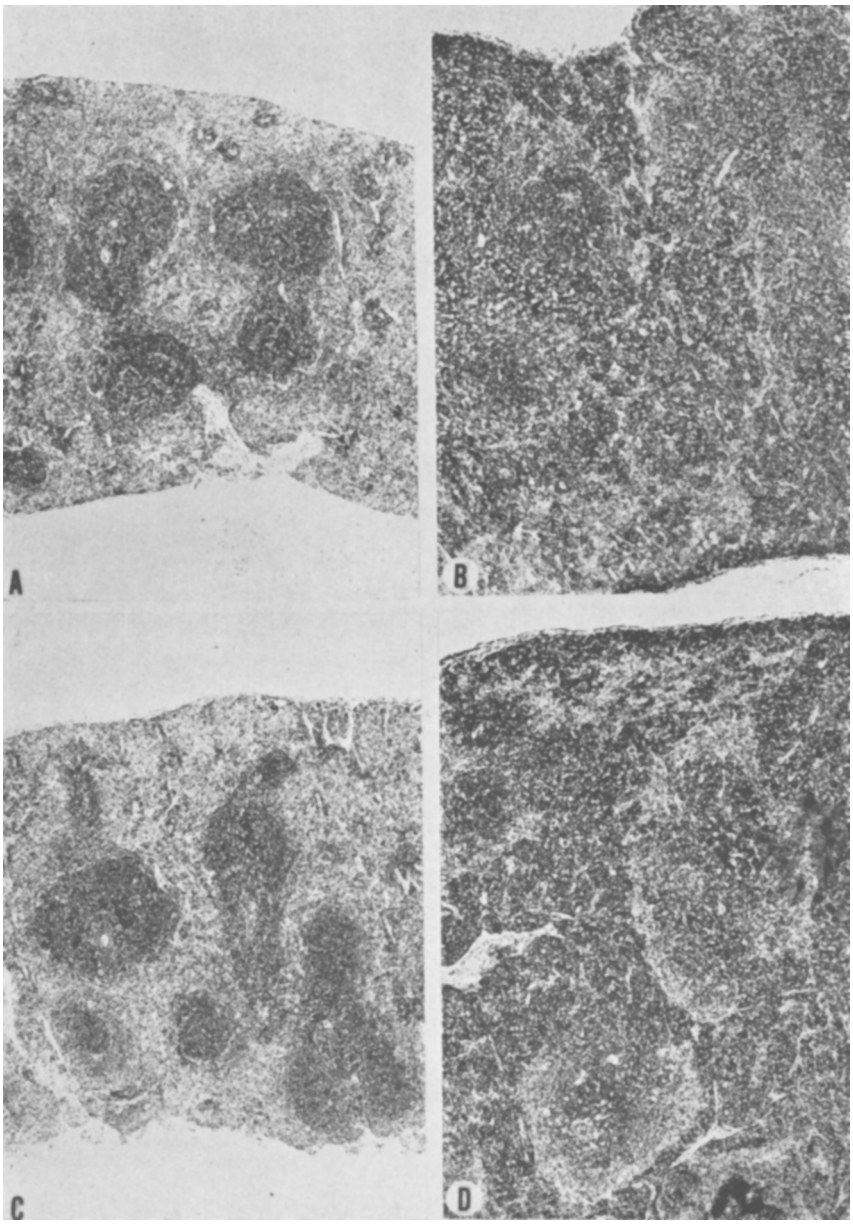

FIG. 7. Histologic effect of 6 0.5-cc injections of anemic rat plasma compared with that of normal rat plasma on splenic erythropoiesis in mice with transfusion-induced polycythemia. Tissues were taken for study 8 days after first inj. of plasma. A. "No injection" polycythemic control spleen. Red pulp is sparsely cellular. B. Spleen of normal plasma-inj. polycythemic mouse. Red pulp is rather heavily populated with erythropoietic tissue. C. Spleen of saline-inj. polycythemic control. Red pulp is sparsely cellular and there are no islands of erythropoiesis. D. Spleen of anemic plasma-inj. polycythemic mouse. There is extreme erythropoietic activity in the red pulp and a moderate reduction in lymphopoietic cells in white pulp.

normal and anemic plasma in hypophysectomized rats and polycythemic mice. It is of considerable importance, however, to note that when one reduces erythropoiesis to an *absolute zero* baseline by transfusion-induced polycythemia, as measured by reticulocytes, Fe^{59} uptake values are 5% or less but never reach a zero baseline. We have demonstrated

in polycythemic animals that the response to an anemic plasma preparation may not be significant by Fe^{59} red cell incorporation studies but that reticulocyte studies clearly indicate activity. On the other hand, as described in Part III(5), when a substantial reticulocyte response occurs, Fe^{59} red cell incorporation clearly differentiates the anemic plasma-injected animals from the controls in the polycythemic recipients, and is equally as sensitive as the hypophysectomized assay preparation.

The slight effect of normal plasma on the reticulocytes of the peripheral blood and the striking effect on splenic erythropoiesis probably represents the effect of a small amount of erythropoietin that is present in normal plasma. Our experience would indicate that one could probably use weight, size, and gross appearance of the spleen of polycythemic mice as a simple index of erythropoietic effect of an unknown plasma. The histologic appearance of the spleen permits differentiation between anemic plasma and normal plasma or saline recipients on the basis of the amount of erythropoiesis in the red pulp. In preliminary studies we have found that normal plasma incubated at 37°C for 20 hours and administered thereafter according to the same dosage schedule as that used for normal or anemic plasma in polycythemic mice fails to increase the number of reticulocytes in polycythemic mice.

In his early studies on transfusions, Robertson(7) described the induction of polycythemia in the rabbit and the effect of this procedure on erythropoiesis. He encountered increasing difficulty in maintaining a polycythemia as transfusion followed transfusion. We have found that it is not difficult to produce and maintain a polycythemia in mice if washed homologous cells are used. However, the repeated injection of heterologous plasma makes the maintenance of a polycythemia more difficult.

Summary and conclusions. 1. Following hypophysectomy of rats, reticulocytes in the peripheral blood fall to a minimal level within a period of 3 to 4 weeks. This reduction represents a 5- to 10-fold decrease from the prehypophysectomy control. This reticulocyte decline is more rapid in 8-week-old rats than in younger animals. 2. Administration of anemic plasma to hypophysectomized rats produces a reticulocyte rise that is many fold greater than that produced by normal plasma or saline. 3. Erythropoiesis, as measured by the number of reticulocytes in peripheral blood, falls to zero in 6 days in mice made polycythemic by repeated intraperitoneal injections of homologous red cells. Anemic plasma obtained from rats or rabbits, when injected intravenously or intraperitoneally into polycythemic mice, produces a reticulocytosis many times greater than that observed from normal plasma. Normal saline produces no increase in reticulocytes. 4. In polycythemic mice and hypophysectomized rats, reticulocyte determinations in the peripheral blood and Fe^{59} red cell incorporation studies are of about equal sensitivity in measuring the response to anemic plasma.

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